



## Species diversity of *Rhinebothrium* Linton, 1890 (Eucestoda: Rhinebothriidea) from *Styracura* (Myliobatiformes: Potamotrygonidae), including the description of a new species

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### Abstract

The present study contributes to the knowledge of the cestode fauna of species of *Styracura* de Carvalho, Loboda & da Silva, which is the putative sister taxon of freshwater potamotrygonids—a unique group of batoids restricted to Neotropical freshwater systems. We document species of *Rhinebothrium* Linton, 1890 as a result of the examination of newly collected specimens of *Styracura* from five different localities representing the eastern Pacific Ocean and the Caribbean Sea. Overall, we examined 33 spiral intestines, 11 from the eastern Pacific species *Styracura pacifica* (Beebe & Tee-Van) and 22 from the Caribbean species *S. schmardae* (Werner). However, only samples from the Caribbean were infected with members of *Rhinebothrium*. *Rhinebothrium tetralobatum* Brooks, 1977, originally described from *S. schmardae*—as *Himantura schmardae* (Werner)—off the Caribbean coast of Colombia based on six specimens is redescribed. This redescription provides the first data on the microtriches pattern, more details of internal anatomy (*i.e.*, inclusion of histological sections) and expands the ranges for the counts and measurements of several features. We describe a new species of *Rhinebothrium* from *S. schmardae* collected off the Caribbean coast of Panama. *Rhinebothrium reydai* n. sp. is diagnosed by possessing four testes per proglottid, acraspedote proglottids, single anterior-most and posterior-most loculi, and bothridia divided into 34–44 loculi. Collectively, these features distinguish the new species from all 41 species of *Rhinebothrium* currently recognized as valid, with the exception of *R. chollaensis* Friggens & Duszynski, 2005. The latter species, a parasite of *Urobatis halleri* (Cooper) from the eastern Pacific Ocean, has a similar morphology in comparison to *R. reydai* n. sp., but can be distinguished by being apolytic instead of eupolytic and by the morphology of the aporal lobe of the ovary, which reaches the mid-lateral margin of the cirrus sac, whereas in *R. reydai* n. sp. the aporal lobe only reaches the posterior margin, since the cirrus sac takes approximately  $\frac{3}{4}$  of the proglottid in width. Also, in *R. reydai* n. sp., the first square proglottid occurs within the anterior third of the strobila (13–30%), whereas in *R. chollaensis* it occurs near the middle of the strobila (42–62%). Further, we discuss the patterns of infection and biogeographical distribution for species of *Rhinebothrium* in species of *Styracura*. The apparent disjunctive distribution of *R. tetralobatum* and *R. reydai* n. sp. in the Caribbean Sea throughout their host distribution, *S. schmardae*, and the absence of species of *Rhinebothrium* in the eastern Pacific sister-host, *S. pacifica*, reveal the importance of sample size and biogeographical representation for documenting the parasite fauna of host lineages.

**Key words:** Cestodes; taxonomy; historical association; geminate species; Caribbean Sea; eastern Pacific Ocean; sample size; patterns of infection; biogeographical distribution

### Introduction

Studies on historical associations of any host-parasite system have the potential to provide useful information for elucidating the origins of the participating lineages (Page & Charleston 1998; Caira & Jensen 2001). The divergence of Neotropical freshwater stingrays (family Potamotrygonidae) from their marine ancestor and the supposed codivergence among cestode parasites and their hosts is a classic example of historical association study (Brooks *et al.* 1981; Blair 1994; Lovejoy *et al.* 1998; Marques 2000). Brooks *et al.* (1981) pioneered the study of this system and, based on parasitological data, suggested that freshwater potamotrygonids derived from a Pacific-

dwelling urotrygonid-like ancestor (referred to as "*Urolophis*" in Brooks *et al.* [1981; pg. 161]) (see also Naylor *et al.* 2012 and Marques & Caira 2016) prior to the uplift of the Andes in the Cretaceous. This seminal study sparked a great deal of debate in the literature (Straney 1982; Caira 1990, 1994; Lovejoy 1997), most of which centered on the apparent lack of robust parasitological data and methods of inference. In recent years, however, a body of evidence has mounted supporting an alternative hypothesis for the sister group of the freshwater potamotrygonids: that the clade comprising the two species of *Styracura* de Carvalho, Loboda & da Silva—previously assigned to amphi-American representatives of *Himantura* Müller & Henle—is the sister group of freshwater potamotrygonids (Aschliman 2011; Naylor *et al.* 2012). Hence, *Styracura* is now considered as a member of the family Potamotrygonidae (de Carvalho *et al.* 2016). The divergence between these two lineages is now thought to be a result of the marine incursion events during the Paleogene period, between the early Miocene and mid-Eocene (*i.e.*, 22.5–46 mya; see Lovejoy *et al.* 1998; Marques 2000; de Carvalho *et al.* 2004). According to the biogeographical hypothesis, the tropical eastern Pacific Ocean and Caribbean Sea represent the hypothetical area of derivation of freshwater potamotrygonids. Hence, the study of the cestode fauna of species of *Styracura* can potentially reveal the historical associations among these related batoid lineages and associated helminth parasites.

The cestode fauna of species of *Styracura* is poorly known despite its relevance for the understanding of this classic biogeographical puzzle. The lack of knowledge is mainly due to the fact that only three studies have reported cestodes from these hosts. Brooks (1977) examined three specimens of *Styracura schmardae* (Werner) from waters off the Caribbean coast of Colombia, from which he described six species of cestodes, namely the onchoproteocephalideans *Acanthobothroides thorsoni* Brooks, 1977, *Acanthobothrium himanturi* Brooks, 1977 and, *Acanthobothrium tasajerasi* Brooks, 1977; and the rhinebothriideans *Anindobothrium anacolum* (Brooks, 1977) Marques, Brooks & Lasso, 2001, *Scalithrium magniphallum* (Brooks, 1977) Ball, Neifar & Euzet, 2003, and *Rhinebothrium tetralobatum* Brooks, 1977. Decades later, Marques *et al.* (1996) examined a single specimen of *S. pacifica* (Beebe & Tee-Van) from waters off Costa Rica, from which they described *Acanthobothroides pacificus* Marques, Brooks & Ureña, 1996 and *Scalithrium geminum* (Marques, Brooks & Ureña, 1996) Ball, Neifar & Euzet, 2003, that they suggested to be “geminant species” (*sensu* Jordan 1908) of *Scalithrium magniphallum* and *Acanthobothroides thorsoni*, respectively. Both studies relied on very few host specimens from but a single, albeit different, locality. In addition, since most of the taxonomic descriptions were based on only a few cestode specimens, the morphological variation in the species was likely underestimated.

Members of the rhinebothriidean genus *Rhinebothrium* Linton, 1890 have been reported from marine and freshwater batoids. The genus possesses the type species of its order, the Rhinebothriidea Healy, Caira, Jensen, Webster & Littlewood, 2009, and other 40 additional valid species. From these species, eight are restricted to freshwater potamotrygonids (Reyda & Marques 2011; Marques and Reyda 2015) whereas only a single species, *R. tetralobatum*, has been reported from one of the two members of the sister clade of the freshwater potamotrygonids: *Styracura schmardae*. To date, no species of *Rhinebothrium* have been reported from *Styracura pacifica*, which is unexpected, since a pair of putative geminant species of *Acanthobothroides* and *Scalithrium* (respectively) have been reported for these sister-species of hosts (as cited above, see Marques *et al.* 1996). Here, we present the results of our most recent survey of *Rhinebothrium* parasitizing species of *Styracura* which includes the redescription of *R. tetralobatum*, the discovery of a second species infecting *S. schmardae*, and a discussion on the patterns of distribution of these parasite species. We also address the potential effect of sample size and biogeographical representation on the accuracy of the documentation of parasite faunas of host lineages (*i.e.*, species and/or clades), leading us to the prediction that at least one additional species of *Rhinebothrium* will likely be found in the future infecting *S. pacifica* in the eastern Pacific Ocean.

## Material and methods

We examined a total of 33 spiral intestines of specimens of *Styracura*, 22 of *S. schmardae* and 11 of *S. pacifica*. Specimens of *S. pacifica* from the eastern Pacific were collected from Panama, Montijo, Veraguas Province [07°29'N, 81°13'W] in January 2015. Specimens of *S. schmardae* were collected from the following four localities: Colombia (near Tasajeras, Magdalena [10°58'N, 74°19'W and 11°0'N, 74°16'W], in May 1989 (five specimens); Belize (Head Caye in Punta Gorda, Toledo [16°13'N, 88°35'W]; North of Southwater Caye and Tobacco Caye in Dangriga, Stann Creek [16°49'N, 88°04'W and 16°54'N, 88°03'W], respectively), in May 2012 (five specimens);

Trinidad and Tobago (Maracas, San Juan-Laventille [10°45'N, 61°26'W], in January 2014 (one specimen); and Panama (western Atlantic Ocean, Almirante, Bocas del Toro province [9°17'N, 82°20'W and 9°17'N, 82°21'W], in January 2015 (11 specimens). The specimens were collected following the guidelines of the permit issued to Janine Caira byINDERENA—Instituto de Pesca y Fauna Terrestre de Bogotá in Colombia and by the Ministry of Forest, Fisheries and Sustainable Development (Belize Fisheries Department—Proc. No 000016-12) in Belize; and to Fernando P. L. Marques by the Ministry of Food Production— Fisheries Division in Trinidad and Tobago (issued on September 30<sup>th</sup>, 2014) and by ANAM—Autoridad Nacional del Ambiente in Panama (SE/A101-14).

After capture, stingrays were euthanized and their spiral intestines removed, opened with a mid-ventral incision, washed with seawater and fixed in a 4% seawater-buffered formalin solution. After a few days, both wash and spiral intestines were transferred to 70% ethanol for long-term storage and subsequent examination. Cestodes recovered from the spiral intestines and its contents were sorted under the stereomicroscope and specimens of *Rhinebothrium* were selected for light and scanning electron microscopy, as well as for histology.

Whole worms selected for light microscopy were hydrated in a regressive alcoholic series, stained with Delafield's hematoxylin (9:1 solution), destained in a 1% acid (HCl) ethanol solution, alkalized in a 1% basic (NaOH) ethanol solution, dehydrated in a progressive ethanol series, cleared in methyl salicylate, and mounted in Canada balsam on glass slides under coverslips. Morphometric data and photographic documentation were obtained using either an Olympus SC30 camera with the Analysis 5.0 software (Olympus Soft Images Solutions) attached to an Olympus BX51 microscope or a Zeiss Axioscope 2 equipped with a SPOT digital camera. The packages Fiji/ImageJ (Schindelin *et al.* 2012) and WormBox (Vellutini & Marques 2014) were used to process images and compute morphometric data, respectively. Only complete specimens with mature (*i.e.*, with open genital pores) or further developed proglottids (*e.g.*, with atrophied testes or vas deferens filled) were examined and measured in this study. All measurements of reproductive structures were taken from terminal proglottids. In cases where the terminal proglottids presented atrophied testes, the data from testes was obtained from subterminal mature proglottids. All measurements are in micrometers unless otherwise stated, and are presented as ranges followed in parentheses by the number of specimens from which the measurement was taken. Repeated measurements for the number and dimensions of testes and for the dimensions of vitelline follicles were averaged for individuals. Terminology for the shape of the bothridia follows Clopton (2004). Line drawings were prepared with the aid of a drawing tube attached to a Zeiss Axioscope 2.

Posterior strobila of specimens selected for histological sections were embedded in paraffin and sectioned at 7µm intervals using a LEICA RM 2025 retracting rotary microtome. Sections were mounted on glass slides flooded with distilled water and initially dried on a slide warmer for 5 minutes and later transferred to an oven for 30 minutes at 60°C. Cross-sections of mature proglottids were stained with Mayer's hematoxylin and counterstained with eosin, dehydrated in a graded ethanol series, cleared in xylene, and mounted in Entellan (Merck). The anterior portion of each worm sectioned was prepared as a whole mount as described above and kept as a voucher.

Scolecocytes selected for scanning electron microscopy (SEM) were carefully cleaned with brushes to remove host tissue and mucus, hydrated in a graded ethanol series, transferred to 1% osmium tetroxide overnight, dehydrated in a graded ethanol series, and placed in hexamethyldisilazane (HMDS). They were allowed to air-dry overnight and were subsequently mounted on carbon tape on aluminum stubs, sputter-coated with gold/palladium and examined with a Zeiss DCM 940 and FEI Quanta 600 FEG scanning electron microscope. The strobila of the worm used for SEM was prepared as a whole mount voucher as described above. Microtriches terminology follows Chervy (2009).

Museum abbreviations are as follows: **HWML**, Harold W. Manter Laboratory, University of Nebraska, Lincoln, Nebraska, U.S.A.; **LRP**, Lawrence R. Penner Parasitology Collection, Department of Ecology and Evolutionary Biology, University of Connecticut, Storrs, Connecticut, U.S.A.; **MZUSP**, Museu de Zoologia da Universidade de São Paulo, Universidade de São Paulo, São Paulo, SP, Brazil; **MIUP**, Museo de Invertebrados G. B. Fairchild, Estafeta Universitaria, Universidad de Panamá, Veraguas, Panama; and **USNPC**, United States National Parasite Collection, Beltsville, Maryland, U.S.A. (now available at the **USNM**, National Museum of Natural History, Smithsonian Institution, Washington, D.C, U.S.A.).

## Results

***Rhinebothrium tetralobatum* Brooks, 1977, Family Rhinebothriidae Euzet, 1953, order Rhinebothriidea (Figs. 1–4)**

**Type host:** *Styracura schmardae* (Werner) de Carvalho, Loboda & da Silva (Myliobatiformes: Potamotrygonidae).

**Type locality:** Caribbean Sea, 15 km west of La Cienaga, Magdalena, Colombia (11°01'N, 74°15'W).

**Additional locality:** Tasajeras, Magdalena, Colombia (10°58'N, 74°19'W and 11°0'N, 74°16'W).

**Site of infection:** Spiral intestine.

**Type material:** Holotype (USNPC 73967) and 6 paratypes (HWML 20253, 20266).

**Voucher specimens deposited:** HWML 110067–110075 (9 complete worms); LRP 9254–9269 (14 complete worms); MZUSP 7926a–7926e and 7927a–7927d (9 complete worms), 7928–7929 (2 SEM vouchers), and 7930a–7930c (voucher and histological sections).

**Prevalence of infection and distribution** – 80% (four of five spiral intestines), seeming to be restricted to the Atlantic coast of Colombia.

**Redescription.** [Based on the type series, and 58 additional mature specimens, which includes 53 whole mounts, two worms observed with SEM, and three prepared as cross-sections]. Worms craspedote (Fig. 1), euapolytic, 4.1–19.0 mm ( $n = 42$ ) long, composed of 80–206 ( $n = 42$ ) proglottids. Scolex 278–801 ( $n = 48$ ) in maximum width, composed of four stalked and bilobed bothridia constricted at center, with muscular rims (Fig. 2A, 3A). Bothridia 487–1,094 ( $n = 48$ ) long by 122–328 ( $n = 48$ ) wide, divided by 23–34 ( $n = 48$ ) transverse septa and one medial longitudinal septum into 47–69 ( $n = 32$ ) loculi; with anterior-most loculus single, 26–50 ( $n = 31$ ) long by 31–59 ( $n = 31$ ) wide; posterior-most loculi double. Cephalic peduncle absent. Proximal and distal surface of bothridia covered with acicular filitriches and gladiate spinitriches (Fig. 3B–G). Anterior portion of strobila covered with capilliform filitriches (Fig. 3H).

Immature proglottids wider than long, becoming longer than wide with maturity, 68–178 ( $n = 42$ ) in number (Fig. 1). Terminal mature proglottids 173–900 ( $n = 41$ ) long by 73–220 ( $n = 42$ ) wide (Fig. 2C,D), 6–62 ( $n = 42$ ) in number. Some terminal proglottids with sperm-filled vas deferens and atrophied testes (Fig. 2D). Testes in anterior  $\frac{1}{3}$  to  $\frac{1}{2}$  of proglottid, oval, 15–44 ( $n = 37$ ) long by 10–32 ( $n = 37$ ) wide, two ( $n = 38$ ) in number, arranged opposite one another (Fig. 2C, 4A). Cirrus sac in anterior  $\frac{1}{2}$  of proglottid, round to pyriform, 25–78 ( $n = 38$ ) long by 28–69 ( $n = 38$ ) wide, containing coiled cirrus; cirrus armed with spinitriches (Fig. 2B). Genital atrium indistinct. Genital pore 18–33% ( $n = 41$ ) of proglottid length from anterior end, irregularly alternating. Vagina opening into genital atrium anterior to cirrus sac, thick-walled, weakly sinuous, somewhat enlarged in proximal part, with antero-medial portion adjacent to cirrus sac, both directed laterally to common genital atrium. Vaginal sphincter absent. Ovary near posterior end of proglottid, bilobed in dorso-ventral view, tetralobed in cross-section (Fig. 4B), symmetrical, 68–348 ( $n = 42$ ) long by 40–112 ( $n = 42$ ) wide at isthmus. Vitelline follicles extending length of proglottid, longer 7–24 ( $n = 15$ ) than wide 4–19 ( $n = 15$ ). Detached gravid proglottids and eggs not observed.

**Remarks.** The original description of *Rhinebothrium tetralobatum* was based on six specimens and did not include information on tegumental structures (*i.e.*, microtriches). In addition, we also detected some discrepancies between the original description and re-examination of the type series. Brooks (1977) reported *R. tetralobatum* as being 15–30 mm in length, with a genital pore position between 44 and 48% from anterior end of proglottid and an ovary measuring 198–300 in length. These accounts differ from our measurements of the type series (*i.e.*, 5.4–9.7 mm, 23–33%, and 86–159, respectively). Furthermore, the examination of additional material shows that *R. tetralobatum* ranges from 4.1 to 19.0 mm in length, possesses genital pores that are located 18 to 33% from the anterior end, and that the ovary is 68–348 in length (Table 1). Also, Brooks (1977) reported the presence of a cephalic peduncle, which was not seen in either the newly collected specimens, or the type series, and described the worms as apolytic rather than euapolytic (*sensu* Caira *et al.* 1999).



**FIGURE 1.** Light micrograph of voucher specimens of *Rhinebothrium tetralobatum* from *Styracura schmardae* (LRP 9255).

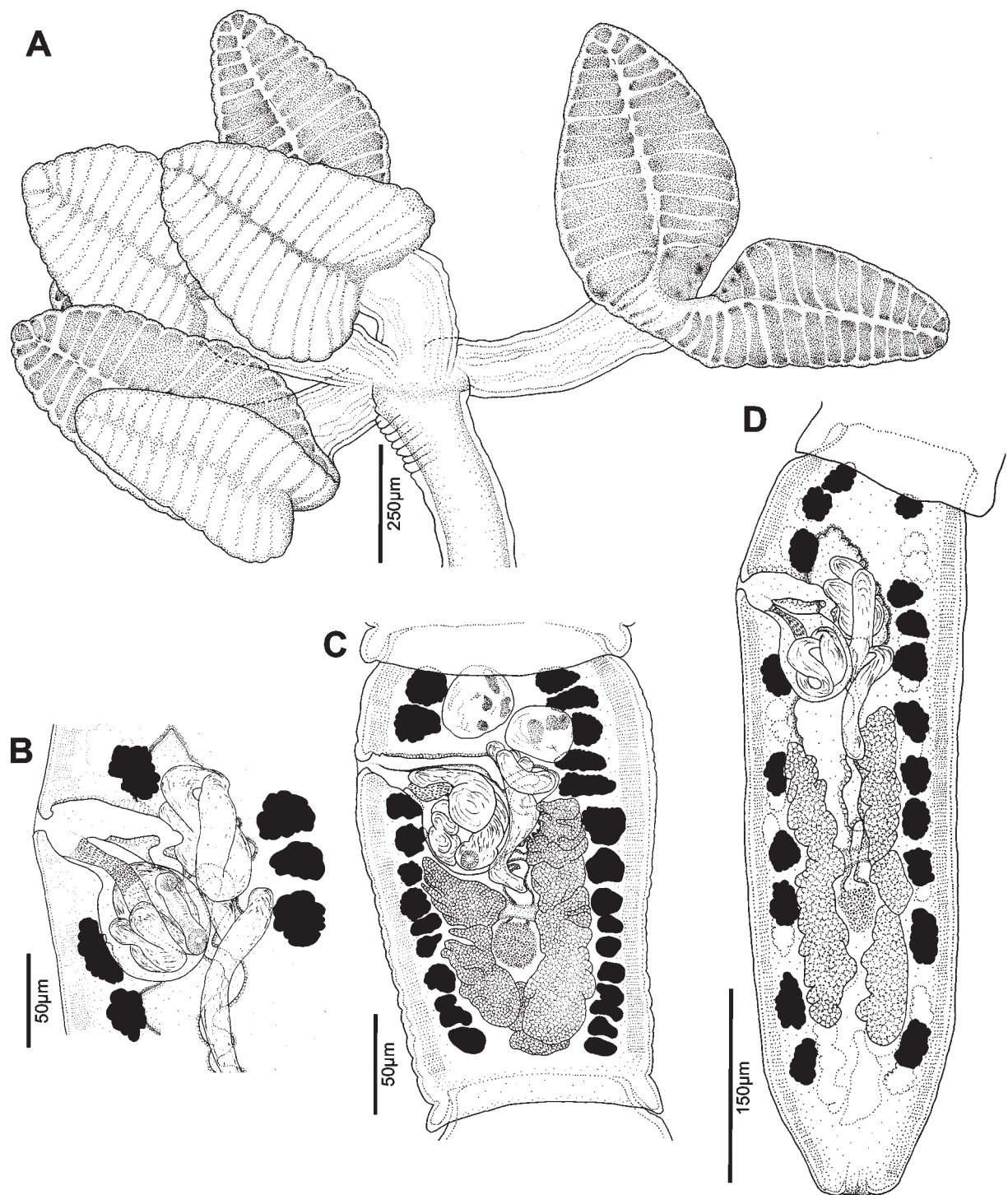


**TABLE 1.** Morphometric and meristic characters of *Rhinebothrium tetralobatum* from the original description and the present study. Measurements are given as the range, followed by number of worms in parentheses and are in micrometers, unless otherwise indicated.

Character	Brooks (1977)	Present study
Total length (mm)	15.0–30.0 (6)	4.1–19.0 (42)
Total number of proglottids	82–100 (6)	80–206 (42)
Scolex width	–	278–801 (48)
Bothridia length	564–804 (6)	487–1,094 (48)
Bothridia width	228–264 (6)	122–328 (48)
Apical sucker length	–	26–50 (31)
Apical sucker width	–	31–59 (31)
Number of loculi	50–54 (6)	47–69 (32)
Number of mature proglottids	–	6–62 (42)
Mature/terminal proglottid length	360–564 (6)	173–900 (41)
Mature/terminal proglottid width	96–156 (6)	73–220 (42)
Terminal proglottid ratio	–	1.6–5.1 (41)
Genital pore position from anterior end	44–48 (6)	18–33 (41)
Number of testes	2 (6)	2 (38)
Testes length	–	15–44 (37)
Testes width	35–58 (6)	10–32 (37)
Cirrus sac length	–	25–78 (38)
Cirrus sac width	50–60 (6)	28–69 (38)
Ovary length	198–300 (6)	68–348 (42)
Ovary width	60–90 (6)	40–112 (42)
Vitelline follicles length	–	7–24 (15)
Vitelline follicles width	30–40 (6)	4–19 (15)

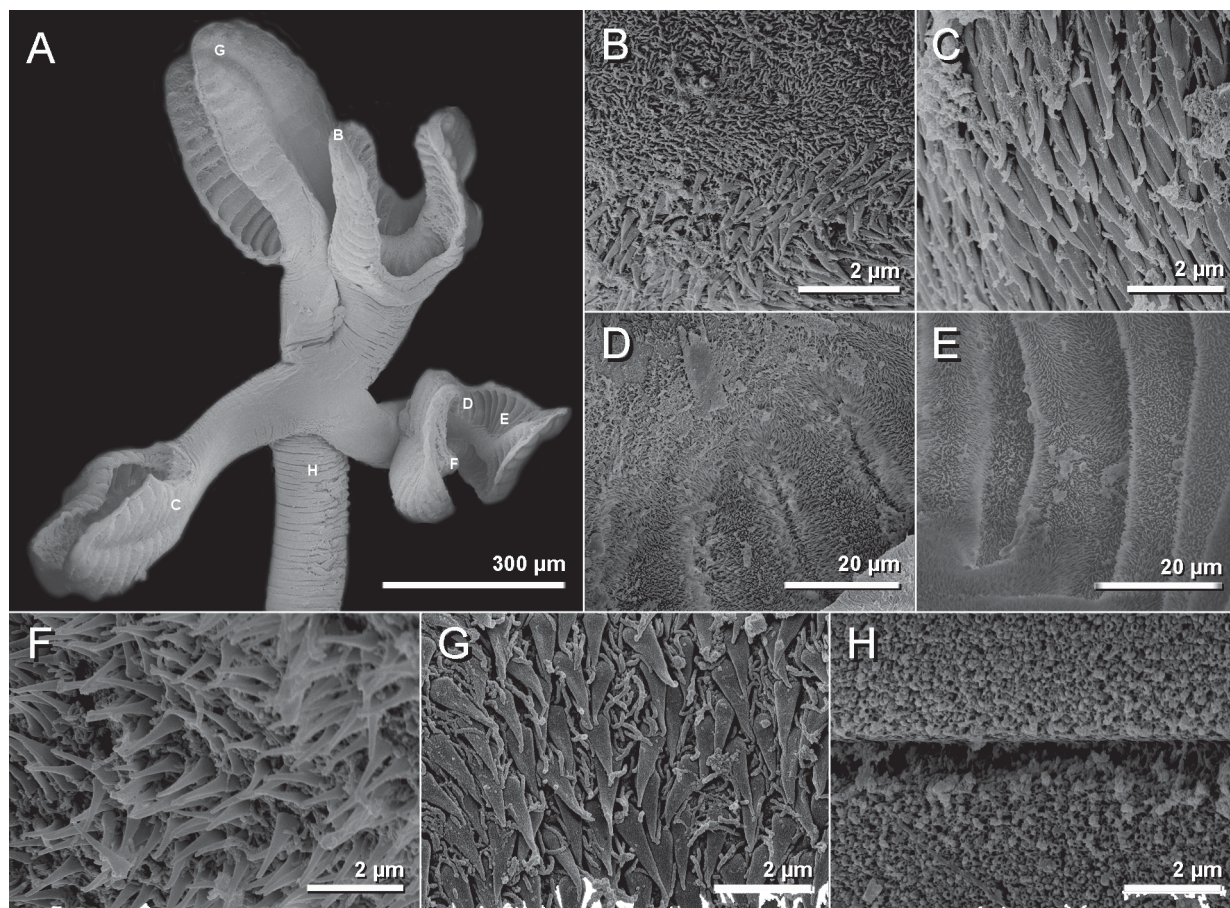
Among 41 valid species of *Rhinebothrium*, *R. tetralobatum* shares the presence of two testes per proglottid with five other members of the genus. This group includes the freshwater species *R. fulbrighti* Reyda & Marques, 2011 ex *Potamotrygon orbignyi* Castelnau from the Bay of Marajó, Brazil; the marine Atlantic *R. biorchidum* Huber & Schmidt, 1985 ex *Urobatis jamaicensis* Cuvier from Jamaica; *R. spinicephalum* Campbell, 1970 ex *Hypanus americanus* (Hildebrand & Schroeder) from the coast of Virginia, U.S.A.; the eastern Pacific *R. ditesticulum* Appy & Dailey, 1977 ex *Urobatis halleri* Cooper from California, U.S.A.; and *R. rhinobati* Dailey & Carvajal, 1976 ex *Rhinobatos planiceps* Garman from the coast Antofagasta, Chile. Biogeographical distribution aside, *R. biorchidum*, *R. rhinobati*, and *R. spinicephalum* differ from *R. tetralobatum* by having fewer segments (15–26, 18–33, 36–49 vs. 80–206, respectively) and a smaller number of bothridial loculi (22–30, 22, 32–34 vs. 47–69, respectively). In addition, *R. biorchidum* and *R. rhinobati* can be further distinguished from *R. tetralobatum* by their smaller size (1.2–2.5, 1.8–2.8 mm vs. 4.1–19.0 mm, respectively).

*Rhinebothrium ditesticulum* and *R. fulbrighti* most closely resemble *R. tetralobatum* in their total length (9.6–28.7 and 3.1–18.0 mm vs. 4.1–19.0, respectively) and the number of proglottids (160–276 and 40–168 vs. 80–206, respectively). Besides the slightly overlap, *R. fulbrighti* possesses fewer bothridial loculi in comparison to *R. tetralobatum* (43–53 vs. 47–69, respectively). In addition to that, the former has been described as having only two ovarian lobes whereas the latter has four. *Rhinebothrium ditesticulum* can be distinguished from *R. tetralobatum* by the morphology of the bothridia. It has a single posterior-most loculus on the bothridia and the anterior and posterior bothridial surfaces are completely separated, whereas, *R. tetralobatum* possess two posterior-most loculi and the anterior and posterior bothridial surfaces are not completely separated, but have a constriction at the center.

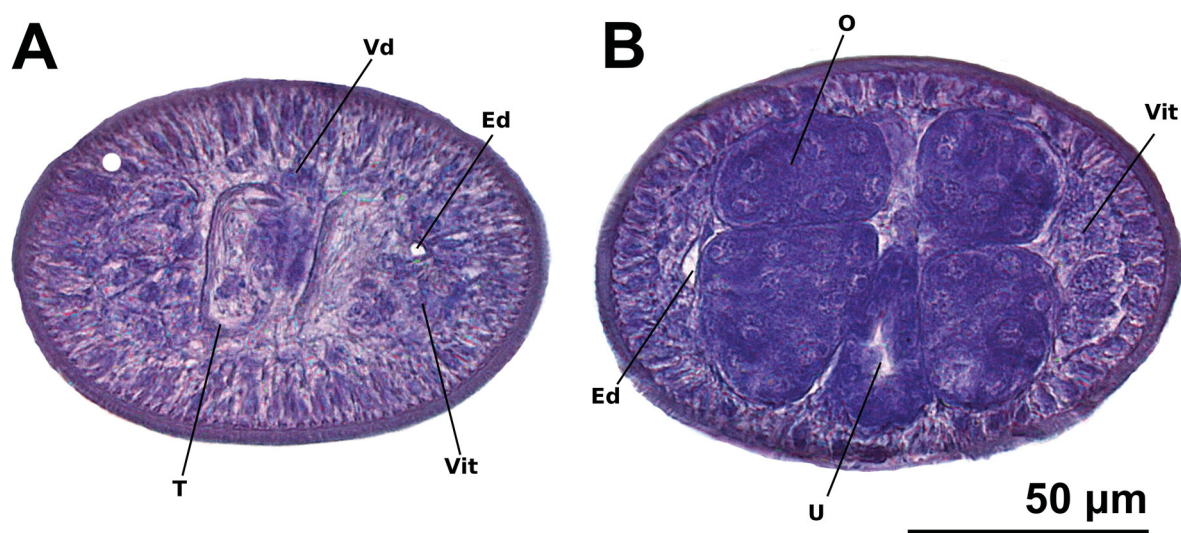


**FIGURE 2.** Line drawings of *Rhinebothrium tetralobatum*. **A.** Scolex (HWML 110067, Voucher), **B.** Cirrus-sac, **C.** Subterminal, mature proglottid, **D.** Terminal, mature proglottid in which testes are atrophied (HWML 110071, Voucher).





**FIGURE 3.** Scanning electron micrographs of *Rhinebothrium tetralobatum* (MZUSP 7928–7929, Vouchers). **A.** Scolex, **B.** Proximal surface of anterior locus, **C.** Proximal surface near centre of bothridium, **D.** Distal surface near centre of bothridium, **E.** Distal surface of transverse septa, **F.** Distal surface near longitudinal septum in anterior region of bothridium, **G.** Proximal surface in posterior region of bothridium, **H.** Surface of anterior portion of strobila.



**FIGURE 4.** Micrographs of transverse histological sections of *Rhinebothrium tetralobatum* (MZUSP 7930a–7930c, Vouchers). **A.** Section at level of testes, **B.** Section at level of ovary. *Abbreviations:* **Ed.** Excretory duct, **O.** Ovary, **T.** Testis, **U.** Uterus, **Vd.** Vas deferens, **Vit.** Vitelline follicle.



***Rhinebothrium reydai* n. sp., Family Rhinebothriidae, order Rhinebothriidea**  
(Figs. 5–8)

**Type host:** *Styracura schmardae* (Werner) de Carvalho, Loboda & da Silva (Myliobatiformes: Potamotrygonidae).

**Type locality:** Caribbean Sea off Almirante, Bocas Del Toro Province, Panama (09°17'N, 82°20'W and 09°17'N, 82°21'W).

**Site of infection:** Spiral intestine.

**Type specimens:** MIUP CR1 (Holotype) and CRP1–CRP9 (Paratypes: 9 complete worms); MZUSP 7931a–7931p (Paratypes: 16 complete worms), 7932 (1 SEM vouchers), and 7933a–7933d (voucher and histological sections); HWML 110076–110082 (Paratypes: 7 complete worms); LRP 9270–9276 (Paratypes: 7 complete worms).

**Prevalence of infection and distribution:** 45.5% (five of 11 spiral intestines), seeming to be restricted to the Caribbean coast of Panama.

**Etymology:** The species is named in honor of Dr. Florian B. Reyda, who took part in the sampling trip to Panama and who has contributed to the knowledge of this group of cestodes for many years.

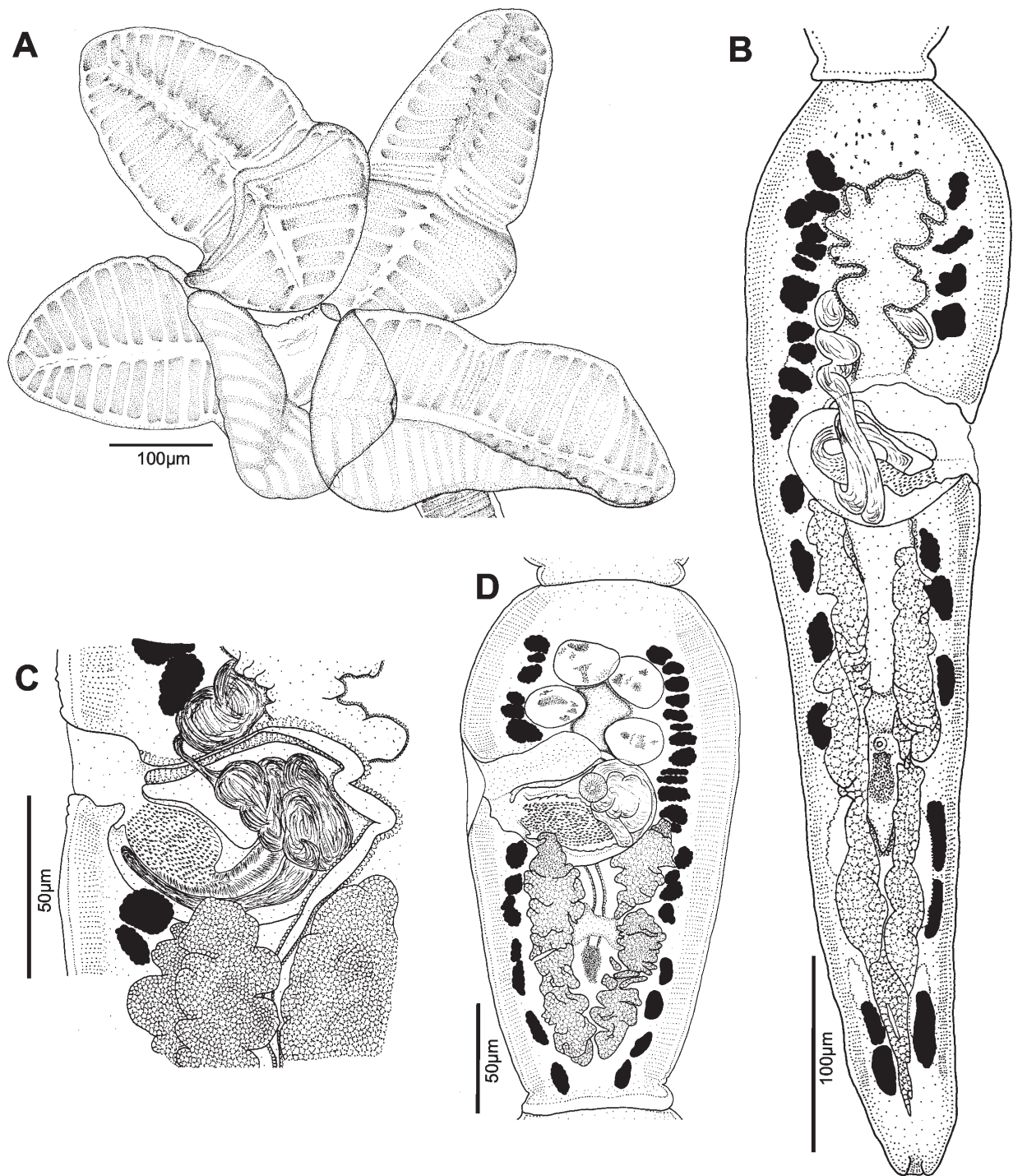
**Description.** [Based on 50 mature specimens: 47 whole mounts, one worm observed with SEM, and two prepared as cross-sections]. Worms acraspedote (Fig. 5), euapolytic, 2.2–7.4 mm ( $n = 47$ ) long, composed of 26–68 ( $n = 47$ ) proglottids. Scolex 237–376 ( $n = 8$ ) in maximum width composed of four stalked and bilobed bothridia constricted at center, with muscular rims (Fig. 6A, 7A). Bothridia 280–461 ( $n = 9$ ) long by 111–202 ( $n = 9$ ) wide, divided by 17–22 ( $n = 15$ ) transverse septa and one medial longitudinal septum into 34–44 ( $n = 15$ ) loculi; anterior-most and posterior-most loculus single. Anterior-most loculus 18–26 ( $n = 10$ ) long by 29–41 ( $n = 10$ ) wide. Cephalic peduncle absent. Proximal and distal surface of bothridia covered with acicular filitriches and gladiate spinitriches (Fig. 7C–G), except for anterior loculus with acicular filitriches only (Fig. 7B). Anterior portion of strobila covered with capilliform filitriches (Fig. 7H).

Immature proglottids wider than long, becoming as long as wide within the anterior third of the strobila (13–30%,  $n = 39$ ) thereafter, longer than wide with maturity, 19–56 ( $n = 47$ ) in number (Fig. 5). Terminal mature proglottids (Fig. 6B, D) 218–554 ( $n = 42$ ) long by 67–146 ( $n = 43$ ) wide, 4–14 ( $n = 47$ ) in number. Some terminal proglottids with sperm-filled vas deferens and atrophied testes (Fig. 6B). Testes in two irregular columns, in anterior  $\frac{3}{4}$  of proglottid, oval, 20–43 ( $n = 27$ ) long by 17–34 ( $n = 27$ ) wide, 4 ( $n = 47$ ) in number (Fig. 6D, 8A). Cirrus sac in anterior  $\frac{1}{2}$  of proglottid, spherical to pyriform in shape, 33–59 ( $n = 39$ ) long by 37–83 ( $n = 39$ ) wide, containing coiled cirrus; cirrus armed with spinitriches (Fig. 6C). Genital atrium prominent. Genital pores 31–49% ( $n = 42$ ) of proglottid length from anterior end, irregularly alternating. Vagina opening into genital atrium anterior to cirrus sac, thick-walled, weakly sinuous, somewhat enlarged in proximal part, with antero-medial portion adjacent to cirrus sac, both directed laterally to common genital atrium. Vaginal sphincter absent. Ovary near posterior end of proglottid, inverted A-shaped in frontal view and tetra-lobed in cross-section (Fig. 8B), symmetrical, 89–316 ( $n = 40$ ) long by 39–79 ( $n = 40$ ) wide at isthmus (Fig. 6B). Vitelline follicles extending length of proglottid, longer 5–15 ( $n = 14$ ) than wide 4–12 ( $n = 14$ ). Detached gravid proglottids and eggs not observed.

**Remarks.** This new taxon is unique among species of *Rhinebothrium* due to a combination of morphological characters, including the presence of only four testes and a single posterior-most bothridial loculus. Compared to other 11 species that share the presence of four testes, only *R. chollaensis* Friggens & Duszynski, 2005 possesses a single posterior-most bothridial loculus. *Rhinebothrium reydai* n. sp. further resembles *R. chollaensis* in total length (2.2–8.4 vs. 1.3–5.1, respectively), number of proglottids (26–68 vs. 32–84, respectively), and number of bothridial loculi (34–44 vs. 40–49, respectively). Both species also have similar bothridial morphology. Friggens & Duszynski (2005) described *R. chollaensis* as having leaf-like bothridia with a slight constriction at the level of the stalk, which is similar to what we observed in *R. reydai* n. sp. . However, after examining images from the type series of *R. chollaensis* (Holotype [USNM 92213] and five paratypes [USNM 92213a–92214d]), we think that the constriction is more pronounced in the former, hence described as bilobed. Despite a similar morphology, *R. chollaensis* is apolytic whereas *R. reydai* n. sp. is euapolytic. Based on the type series, both species differ on the morphology of the aporal lobe of the ovary, which reaches the mid-lateral margin of the cirrus sac in *R. chollaensis*, whereas in *R. reydai* n. sp. the aporal lobe only reaches the posterior margin, since the cirrus sac takes approximately  $\frac{3}{4}$  of the proglottid in width. Also, in *R. reydai* n. sp. the first square proglottid occurs within the anterior third of the strobila (13–30%,  $n = 39$ ), whereas in the type series of *R. chollaensis* it occurs near the middle



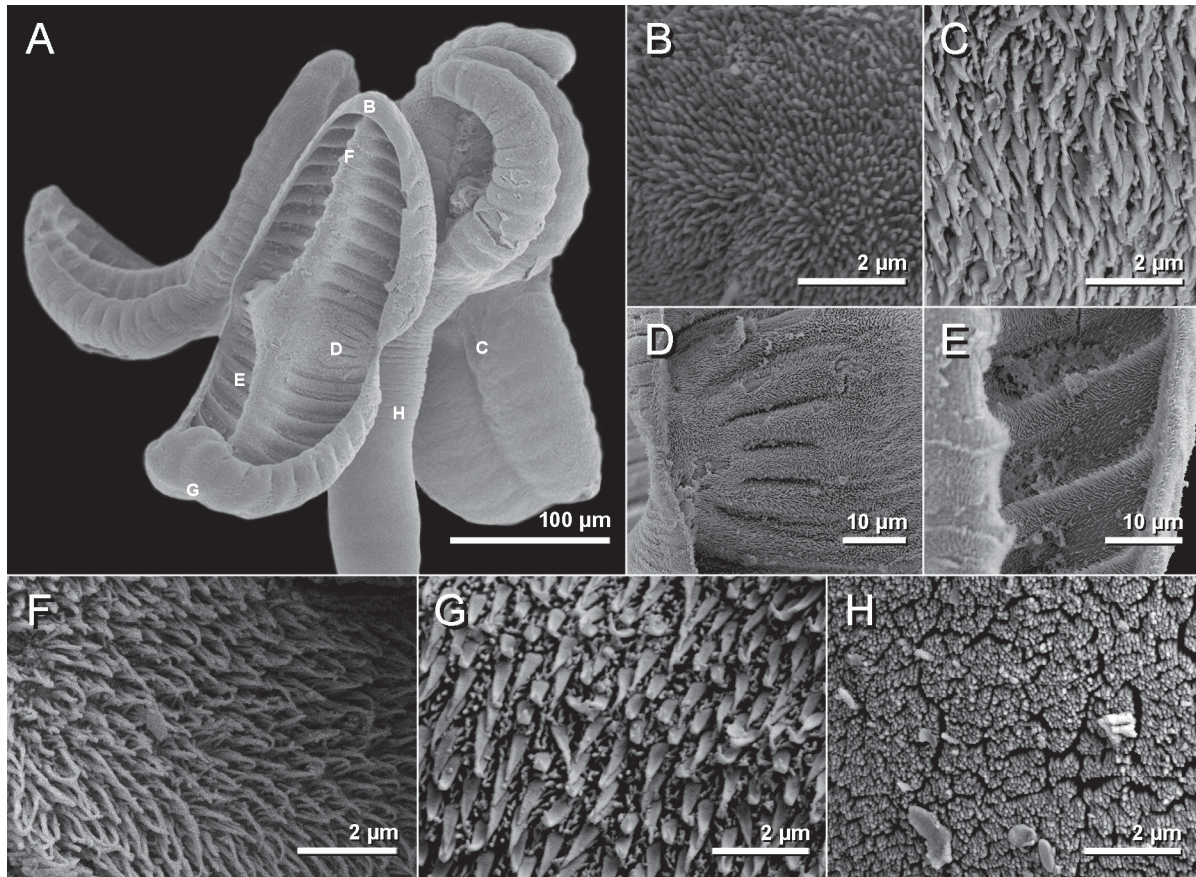
**FIGURE 5.** Light micrograph of the holotype of *Rhinebothrium reydai* n. sp. from *Styracura schmardae* (MIUP CR1).



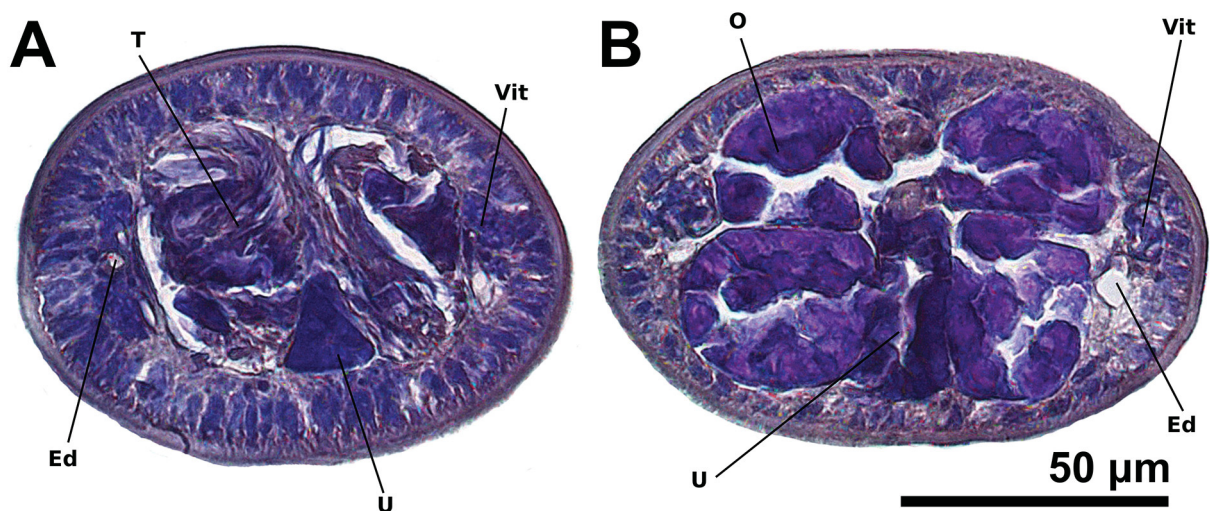
**FIGURE 6.** Line drawings of *Rhinebothrium reydai* n. sp. **A.** Scolex (MIUP CR1, Holotype), **B.** Terminal, mature proglottid in which testes are atrophied (MZUSP 7931b, Paratype), **C.** Cirrus-sac (MZUSP 7931p, Paratype), **D.** Subterminal, mature proglottid (MZUSP 7931b, Paratype).

of the strobila (42–62%,  $n = 6$ ). The morphological resemblance between these two species and their biogeographical distributions (*i.e.*, *R. chollaensis* from the eastern Pacific Ocean vs. *R. reydai* n. sp. from the western Atlantic Ocean), might also suggest a close phylogenetic association, which has yet to be tested within the context of a phylogenetic study.





**FIGURE 7.** Scanning electron micrographs of *Rhinebothrium reydai* n. sp. (MZUSP 7932, Paratype). **A.** Scolex, **B.** Proximal surface of anterior loculus, **C.** Proximal surface near centre of bothridium, **D.** Distal surface near centre of bothridium, **E.** Distal surface of transverse septa, **F.** Distal surface near longitudinal septum in anterior region of bothridium, **G.** Proximal surface in posterior region of bothridium, **H.** Surface of anterior portion of strobila.



**FIGURE 8.** Micrographs of transversal histological sections of *Rhinebothrium reydai* n. sp. (MZUSP 7933a–7933d, Paratypes). **A.** Section at level of testes, **B.** Section at level of ovary. *Abbreviations:* **Ed.** Excretory duct, **O.** Ovary, **T.** Testis, **U.** Uterus, **Vd.** Vas deferens, **Vit.** Vitelline follicle.

## Discussion

Studies of host-parasite co-evolution require accurate taxonomic information to establish precise associations among lineages (Caira & Jensen 2001; Paterson and Banks 2001). Within this context, the main goal of the present study was to document the fauna of *Rhinebothrium* from species of *Styracura* with the expectation that marine lineages closely related to the freshwater lineages of *Rhinebothrium* found in freshwater potamotrygonids will be identified in future studies. Brooks (1977) examined three specimens of *S. schmardae* off the coast of Colombia (see Thorson *et al.* 1983) and described six species of “tetraphyllideans”, including *R. tetralobatum*. Marques *et al.* (1996) examined a single specimen of *S. pacifica* from the coast of Costa Rica from which they described *Acanthobothroides pacificus* and *Scalithrium geminum*. The authors considered these two species from *S. pacifica* to be putative geminate species of *A. thorsoni* and *S. magniphallum*, both found parasitizing the host sister-species *S. schmardae*. Therefore, our study started with the premise that at least one new species of *Rhinebothrium* would be present in the eastern Pacific coast of Panama.

Surprisingly, *Rhinebothrium* was not found parasitizing the Pacific host, *S. pacifica*, despite the common pattern of pairs of transisthmian species cited above for cestodes and reported for many other lineages of Metazoa (Marques *et al.* 1996; Lessios 2008; O'Dea *et al.* 2016). In addition, since members of *Rhinebothrium* are commonly found in batoids (Linton 1890; Brooks 1977; Friggens & Duszynski 2005; Reyda & Marques 2011; Marques & Reyda 2015; Golestaninasab & Malek 2016), we assume that the lack of members of this genus in this host could be an artifact of sample size and/or biogeographical representation. Therefore, we expect that future studies with new samples from the eastern Pacific Ocean will reveal the existence of at least one member of *Rhinebothrium* infecting *S. pacifica*.

Species of *Rhinebothrium* of *S. schmardae*, including the absence of members of the genus in the host population in Belize, revealed that the parasite diversity is heterogeneously distributed throughout the range of the host. A total of five specimens of *S. schmardae* from Belize were examined, including reasonably large specimens (*e.g.*, with a disk diameter larger than 75 cm) that were highly infected with cestodes, and yet no specimens of *Rhinebothrium* were found. Although the number of hosts sampled in Belize ( $n = 5$ ) could be considered suboptimal, we would expect to detect the presence of species of *Rhinebothrium* with a confidence of 95%, if the prevalence of member of this genus is above 45% (see Post & Millest 1991). Albeit this estimate is based on a series of assumptions of sampling design and parasite distribution among host individuals (most likely not met for our samples), it might help us to understand the apparent absence of species of *Rhinebothrium* in that population. The prevalence of the new species *R. reydei* in the host population of Panama was 45.5% (*i.e.*, five out of 11 specimens infected), whereas for *R. tetralobatum* it was 80% (*i.e.*, four out of five infected) in Colombia. These values suggest that the prevalence of species of *Rhinebothrium* in some populations of *S. schmardae* are above the levels that would allow the detection of members of this genus (if present) in Belize, assuming that the prevalence of *Rhinebothrium* in this population followed the same trend observed (*i.e.*,  $\geq 45.5\%$ ). Theoretically, given the number of hosts we examined from Belize ( $n = 5$ ), it would be likely that we would not find any species of cestodes with prevalence below 45.5%. Although all these estimates are based on a small number of samples and simplified models, the data at hand suggests a considerable heterogeneity in cestode composition in species of *Styracura*.

Heterogeneity in parasite composition among hosts is known to radically alter the shape of species accumulation curves (Dove & Cribb 2006) imposing constraints to properly assess the parasite richness of host lineages. Species accumulation curves plot species discovery as a function of sampling effort. This concept is well known by community ecologists within and outside the parasitological literature (Goteli & Colwell 2001; Dove & Cribb 2006; Kamiya *et al.* 2014; Poulin 2014). However, we are unaware of studies that considered species accumulation curves where the main goal was to determine the richness of parasite lineages for addressing historical associations. This should be as relevant in co-evolutionary studies as it is in community ecology of parasites, since species accumulation curves approaching an asymptote should indicate that sampling effort was adequate to characterize the parasite lineages associated with a given host.

How species richness is distributed among sampling units and between scales of organization is generated by heterogeneity in parasite composition (Dove & Cribb 2006). Hence, if richness is concentrated at the individual sample level, that is higher  $\alpha$ -richness, the parasite composition would be less heterogeneous and species accumulation curves would approach the asymptote faster compared to communities dominated by  $\beta$ -richness. In the latter, individual host fauna tends to be dissimilar and total richness is only increased by a regional pool host



samples. For communities with higher  $\beta$ -richness, species accumulation curves tend to be characterized by gradual slopes and late asymptotes, thereby depending on a greater sampling effort. This might be the case for the cestode richness of species of *Styracura*.

The only example we are aware of that reports heterogeneity in parasite composition along batoid host range was provided by Mojica *et al.* (2014). These authors addressed the richness of lecanicephalids of the genus *Hornellobothrium* Shipley and Hornell, 1906 parasites of *Aetobatus ocellatus* Kuhl from Borneo and Northern Australia. They found this host to house only one species of the genus in each locality surveyed (*e.g.*, *H. extensivum* Jensen, 2005 from the Timor Sea; *H. gerdaae* Mojica, Jensen & Caira, 2014 from the Gulf of Carpentaria; *H. najaforme* Mojica, Jensen & Caira, 2014 from the Arafura Sea). Mojica *et al.* (2014) credit the pattern to be an artifact of sampling across the Indo-Pacific localities, since for each of them only a single host specimen was examined. In theory, only parasites with prevalence higher than 95% would be detected with 95% confidence with the examination of a single host specimen (Post & Millest 1991). We are unaware of any other example within batoids in which a widespread host has been examined for cestodes along its entire range. Therefore, we are yet to understand how  $\alpha$ - and  $\beta$ -richness shape the total species richness of cestodes in batoid fishes. Nonetheless, it is evident that a well-designed study, accounting for sample size bias and ideal biogeographical representation would be important to understand the patterns that have been observed for lecanicephalids in *Aetobatus ocellatus* from the Indo-West Pacific and species of *Rhinebothrium* from *S. schmardae* in the Caribbean.

We suggest that species accumulation curves should be considered beyond parasite ecology studies. Their relevance to co-evolutionary studies rests on the importance of sample size and biogeographical representation to document the lineages associated with a given host. We have to keep in mind that there are additional components of the hierarchical structure of communities that might influence the shape of species accumulation curves, which might even require additional sampling effort. In general, we tend to assume that individual hosts are the main unitary measure of sampling effort. This uncritical assumption ignores that hosts in a population differ in any number of ways including size, age, diet and behavior. Those differences might influence local richness and diversity. For instance, there is some evidence that elasmobranchs host a different cestode fauna as their mouth size and diet changes throughout their life (Caira 1990; Caira & Euzet 2001).

The results suggest that we have to rethink the how we approach sampling design for co-evolutionary studies by incorporating the role of  $\alpha$ - and  $\beta$ -richness on the composition of cestode fauna target hosts. We predict that the patterns of parasite distribution we observed in species of *Styracura* are likely to be replicated for other batoids as we compile information from different host lineages. If  $\beta$ -richness is a relevant component of total richness, as it seems to be, we will have to allocate additional time and resources to recognize the parasite lineages associated with host lineages. Within this framework, we predict that as we increase our sample size along the distribution of both species of *Styracura*, we will certainly find additional species of cestodes, especially in the tropical eastern Pacific.

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